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Highly efficient synthesis of oligodeoxyribonucleotides using α-phenyl cinnamoyl group for selective amino protection

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ABSTRACT

α-phenyl cinnamoyl (α-PhCm) group has been found to be highly selective for exocylic amino function of all the three deoxynucleosides viz, 2'-deoxyadenosine, 2'-deoxyguanosine and 2'-deoxycytidine. The stereospecific nature of the group confers stability to the N-protected derivatives of 2'-deoxyadenosine and 2'-deoxyguanosine towards acids thereby minimising depurination. The easy preparation and introduction of the group, stability of the protected monomers, milder conditions for deprotection resulting in negligible side products during synthesis and above all hydrophobicity of the group are the additional advantages.

INTRODUCTION

The most important aspect of the chemical synthesis of oligodeoxyribonucleotides is the selective protection and deprotection of different nucleophilic sites of monomer building blocks. The amino protecting groups remain intact throughout the course of synthesis, therefore are extremely important. Several groups have been used for the protection of amino function (1−7) but selective protection of amino function is still a challenging and important problem. Although attempts have been made from time to time to achieve selective protection but notable success has only been achieved in case of 2'-deoxycytidine (8,9). In our previous communications (10−12) we have reported some efforts in this direction, which have met with partial success. Jones procedure is an alternate one whereby prior silylation of hydroxy functions is employed (13).

In case of α-phenyl cinnamoyl group (α-PhCm) we have found that it is highly selective for exocyclic amino function of 2'-deoxyadenosine, 2'-deoxyguanosine and 2'-deoxycytidine with negligible reaction with sugar hydroxyls. With none of the groups so far reported in literature, such selectivity has been reported. Moreover, it is remarkable that it is selective for all the three deoxynucleosides and simultaneously reactive enough to yield the N-protected derivatives in fairly good yields. A hexamer d(AAGCTT) and a dodecamer d(ACCACCACCACC) have been synthesized in fairly good yields using this group by phosphotriester and phosphoramidite approaches respectively on solid support, thereby proving the efficiency and suitabilty of the group for oligonucleotide synthesis.

In the conventional routes of oligonucleotide synthesis pixyl (Px) and dimethoxytrityl (DMTr) groups at 5'-end are removed by acid treatment which causes depurination at each coupling step thereby lowering the overall yield (14,15). To overcome this problem several attempts were made to remove pixyl and dimethoxytrityl group by alternative reagents (16,17) or some base labile groups for 5'-OH function have also been reported (18). An

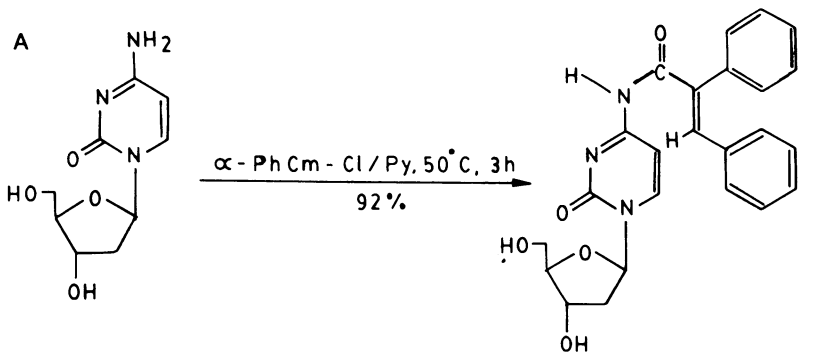

Fig.1 (A) Selective protection of amino group on 2'-dC

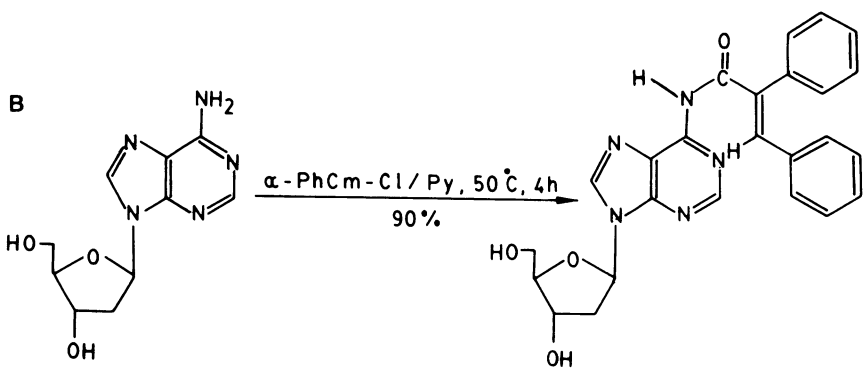

Fig.1 (B) Selective protection of amino group on 2'-dA

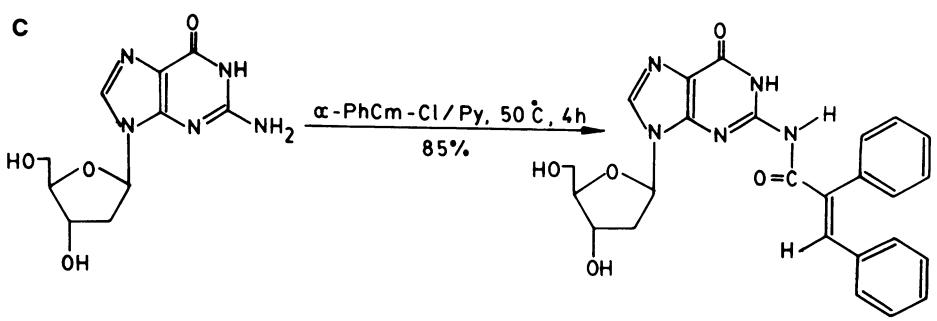

Fig.1 (C) Selective protection of amino group on 2'-dG

Table 1. Yield and characterisation of α-phenyl cinnamoyl protected dC,dA and dG.

Compound	Yield	R_f	UV(CH_2Cl_2) λmax (nm)	λmin (nm)	IR_{KBr} νmax cm^{-1}	Elemental analysis
N^4-α-PhCm-dC	92%	0.41[b]	304 256 232	288	3500−3000 (S,B) 1720(S),1625(S), 1500(S),1320(M),1240(S) 1100(M), 720(W)	$C_{24}H_{21}O_5N_3$ (431) Calcd.C,66.82%;H,4.87%; N, 9.74%. Found C,66.85%;H,4.82%; N, 9.72%
N^6-α-PhCm-dA	90%	0.48[b]	288	250	3500−3400(S,B),3000(S) 1750(S),1632(S),1500(S), 1350(M),1200(S),768(W)	$C_{25}H_{21}N_5O_4$ (455) Calcd.C,65.93%;H, 4.61%; N,15.39% Found C,65.88%;H, 4.63%; N, 15.35%
N^2-α-PhCm-dG	85%	0.43[b]	284	246	3460−3400(S,B),3000(S) 1705(S),1665(S),1500(S), 1300(M),1180(S),1020(W) 690(S)	$C_{25}H_{21}N_5O_5$ (471) Calcd.C, 63.69%;H,4.45%; N, 14.86% Found C,63.64%;H, 4.46%; N, 14.84%

S (Sharp), M (Medium), W (Weak), B (Broad)
[b] (Solvent, CH_2Cl_2/CH_3OH 9.5:0.5 v/v)

alternative solution is to use some exocyclic amino protecting groups which render acid resistivity to the glycosyl bond.

In our earlier publications we have reported three new groups viz. 3-methoxy-4-phenoxybenzoyl (10) phenoxyacetyl (11) and naphthaloyl (12) as depurination resistant groups. Some other groups have also been reported to be less susceptible to the protic acid depurination (2−4; 19−21). N-α-phenyl cinnamoyl derivatives of 2′-deoxyadenosine and 2′-deoxyguanosine have been found to be better depurination resistant than other derivatives reported so far.

MATERIALS AND METHODS

All the four deoxynucleosides, 5′-O-dimethoxytrityl N^6-benzoyl-2′-deoxynucleosides-3′-β-cyanoethyl-N, N-diisopropyl phosphoramidites, tetrazole, dimethoxytrityl chloride, mesitylene sulphonyl-3-nitro-1,2,4 triazole (MSNT), 1,1,3,3-tetramethyl guanidine and 4-nitrobenzaldoxime were purchased from Cruachem Co. and DCC, 1-methyl imidazole, phenylacetic acid and ninhydrin from Sigma Chem. Co.

α-phenylcinnamic acid was prepared by Perkin reaction involving phenylacetic acid, benzaldehyde and acetic anhydride in presence of triethylamine as catalyst (22).

LCAA/CPG was used as polymer support. DNA bench synthesiser (Omnifit Ltd. Cambridge) and Gene assembler (Pharmacia) were used to synthesize sequences by phosphotriester and phosphoramidite approaches respectively. Purification was done on mono 'Q' anion exchange column. Hitachi 220S spectrophotometer was used for U.V. measurement and elemental analysis was done on the analyser Carlo Erka 1106.

Solvents were duly purified and dried before use. Pyridine was refluxed with ninhydrin and distilled over KOH. Silica gel G (Merck) plates were used for tlc. Plates were sprayed

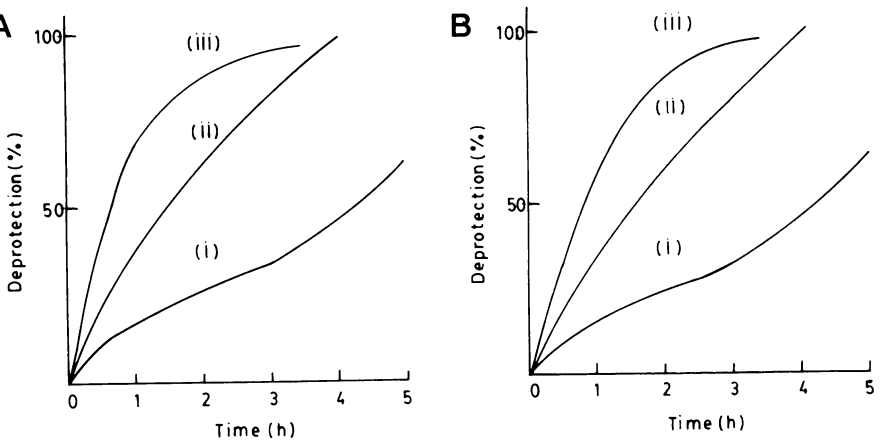

Fig.2 (A) Deprotection of N-α-Phenyl Cinnamoyl 2'-deoxycytidine with 40% ammonia at (i) 30°C (ii) 40°C (iii) 50°C

Fig.2 (B) Deprotection of N-α-phenyl Cinnamoyl 2'-deoxyadenosine with 40% ammonia at (i) 30°C (ii) 40°C (iii) 50°C

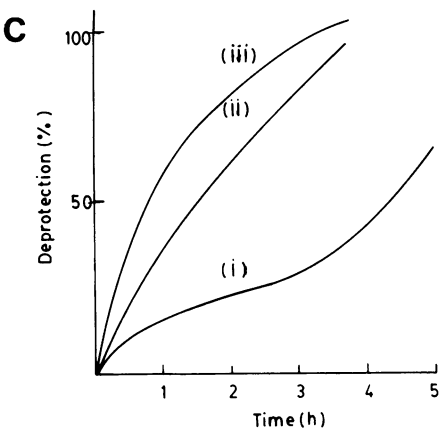

Fig.2 (C) Deprotection of N-α-phenyl Cinnamoyl 2'-deoxyguanosine with 40% ammonia at (i) 30°C (ii) 40°C (iii) 50°C

with iodine, sulphuric acid and perchloric acid for location and differentiation of different products. Small amounts of all the three new derivatives [Figure 1,(A), (B) & (C)] were hydrolysed to get the starting nucleosides for confirmation of their structures (23). All the tlc analysis were done using solvent system a; (CH_2Cl_2/CH_3OH;9.8: 0.2 v/v), b; (CH_2Cl_2/CH_3OH; 9.5:0.5 v/v) and c; (CH_2Cl_2/CH_3OH; 9:1 v/v).

Preparation of α-Phenyl Cinnamoyl Chloride

α-phenyl cinnamic acid (22.4 g, 0.1 mmol) and phosphorous pentachloride (19.8 g, 0.1 mmol) were taken in a 100ml R.B. flask fitted with a reflux condenser and a $CaCl_2$ guard tube which was connected to a gas absorption device. The reaction mixture was heated on a waterbath for 2 h. A pale yellow homogenous liquid was formed. Excess of $POCl_3$

Table 2. Preparation and characterisation of 5',N-protected units.

Derivatives	Reaction time (h)	Yield (%)	R_f	$\lambda max^{(nm)}$
5'-O-DMTr-N^4-α-PhCm-dC	2.0	90	0.82[a]	228,262,308
5'-O-DMTr-N^6-α-PhCm-dA	2.0	92	0.76[a]	286
5'-O-DMTr-N^2-α-PhCm-dG	2.5	95	0.73[a]	288

a, (Solvent CH_2Cl_2/CH_3OH 9.8:0.2 v/v)

was distilled out at ordinary pressure by heating in an oil bath and raising the temperature gradually to 125°C. The remaining portion was collected at 45°C/0.2 mm Hg.

This fraction was characterised as α-phenyl cinnamoyl chloride by comparing it with corresponding acid chromatographically and spectroscopically. It was stored in a sealed glass phial to protect it from moisture. The yield of the chloride was 12 ml (50%).

General method for the preparation of N-α-phenylcinnamoyl-2'-deoxynucleosides

Each deoxynucleoside (1 mmol) was dried by evaporation *in vacuo* with pyridine (3×4 ml). It was then suspended in dry pyridine (10 ml) and α-phenylcinnamoyl chloride (1 mmol) was added. The flask was sealed and shaken in dark at 50°C for 4 h. A clear yellow solution was obtained.The reaction mixture was evaporated to a gum *in vacuo*. It was then poured into 5% aq. $NaHCO_3$ solution and extracted with dichloromethane (4×5 ml). Organic layer was washed with water,dried over Na_2SO_4 and filtered. The combined dichloromethane solution was concentrated to a minimum and was loaded over silica gel column. The elution was done with CH_2Cl_2/CH_3OH in increasing polarity. The desired fractions were pooled, evaporated to dryness, dissolved in minimum amount of dichloromethane and the product was precipitated by gradual addition of it into rapidally stirred hexane (60−80°C). The product thus obtained was filtered and dried. Data obtained are tabulated in (Table 1).

Conditions for removal of α-phenyl cinnamoyl group

The removal conditions for α-phenyl cinnamoyl group in case of all the three deoxynucleosides were studied by treating the N-protected derivatives with 40% ammonia at 30°, 40° and 50°C. Reactions were quenched after 0.5, 1, 2, 3, 4, and 5 h duration. After hydrolysis, the mixtures were analysed for deprotected 2'-deoxynucleosides on semipreparative tlc and subsequent estimation by U.V. spectroscopy.40% ammonia at 40°C for 4 h was found to be the optimal condition for the removal of the group (cf, benzoyl group; Conc. NH_3 for 5 h at 50°C) though the initial rate of hydrolysis was different in each case. [Figure 2, (A), (B), & (C)]

General method for preparation of 5'-0-dimethoxytrityl-N-α-phenyl cinnamoyl-2'-deoxynucleosides (5'-0-DMTr-N-α-PhCm-dNS)

Each N-α-phenyl cinnamoyl 2'-deoxynucleoside (1 mmol) was treated with 4,4'-dimethoxytrityl chloride (1.2 mmol) in pyridine (10 ml) in presence of 4-dimethylamino pyridine as catalyst at r.t. (25−30°C) to get its trityl derivative. After completion of the reaction (as checked by tlc), the clear solution was evaporated to a gum *in vacuo* and worked up by the usual procedure (24). Yield, R_f and λ_{max} of different derivatives are shown in (table 2).

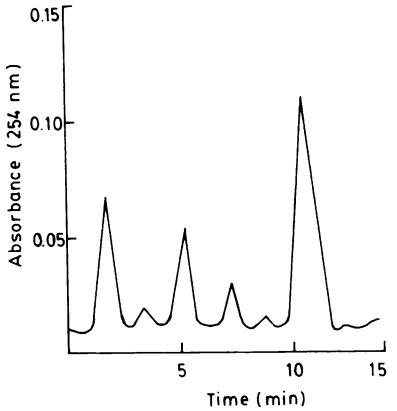

Fig.3 Ion exchange hplc profile of Crude hexamer d(AAGCTT), gradient 0–50% buffer B,13 min,temp 25°C.

General method for the preparation of triethylammonium [5'-O-dimethoxytrityl-N-α-phenylcinnamoyl- 2' -deoxynucleosides-3'-O-(2-chlorophenyl phosphate)]

Each 5', N-protected nucleoside (0.6 mmol) was treated with freshly prepared solution of 2-chlorophenyl phosphoroditriazolide (1.2 mmol) in dry THF (5 ml). Reaction mixtures were worked up by the usual procedure (24) and the product was used as such for further couplings.

Preparation of 5'-O-4,4'-dimethoxytrityl-N^4-α-phenyl cinnamoyl-2'-deoxycytidine-3'-O-succinate

5'-O-4,4'-dimethoxytrityl-n^4-α-phenylcinnamoyl–2'-deoxycytidine (0.5 mmol) dissolved in pyridine (4 ml) was treated with succinic anhydride (0.5 mmol) containing 4-dimethylamino pyridine (0.25 mmol) . Reaction mixture was stirred for 12 h at r.t. (25–30°C) and worked up as usual(24).The product was obtained in 72% yield R_f;0.3,solvent,C.

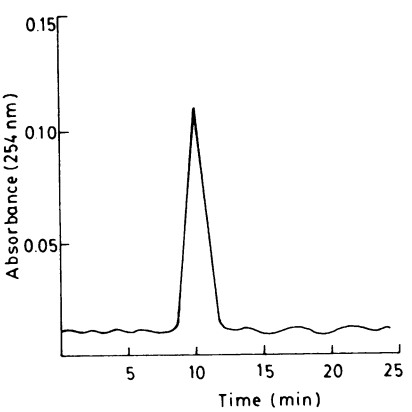

Fig.4 Ion exchange hplc profile of dodecamer d(ACCACCACCACC) gradient 0–70% buffer B,25 min, temp 25°C after purification on reversed phase C_{18} SEP-PAK Cartridge.

Preparation of hexamer d(AAGCTT) by phosphotriester approach

5′-O-4,4′-dimethoxytrityl thymidine derivatised LCAA/CPG (100mg) was taken in the column of 'DNA-bench Synthesiser'. After usual wash cycle (24) a solution of triethylammonium salt of 5′-O-dimethoxytrityl thymidine-3′-O-2-chlorophenyl phosphate (25 μmole) in anhydrous pyridine (0.2 ml), freshly activated with MSNT (137 μmole) and 1-methyl imidazole(0.01 ml)was injected onto the column and coupling time was allowed for 15 min.After second wash cycle,similar couplings were effected respectively with triethylammonium salt of 5′-O-DMTr-dC-3′-O-2-chlorophenyl phosphate, triethylammonium salt of 5′-O-DMTr-dG-3′-O-2-chlorophenyl phosphate and the analogous salts of protected dA in duplicate.The coupling yield determined by trityl estimation was found to be more than 95%.After the fifth coupling resin was washed with pyridine, dichloromethane and finally with ether and dried.

The hexamer linked to the support was deprotected respectively by treatment with (i) 1,1,3,3-tetramethyl guanidine and 4-nitrobenzaldoxime,(ii) 40% ammonia and (iii) 80% acetic acid.The aqueous solution of the hexamer was washed with ether (5×3 ml) and taken up in water for purification.

A gradient hplc system was set up using Mono 'Q' anion exchange column (5×50 mm) with 10 mM NaOH , pH 12 and 10 mM NaOH,pH 12 with 1.5 M NaCl as buffer A and buffer B respectively. Fractions were monitored at 254 nm and pooled. Fraction containing hexanucleotide was desalted by passing it through Biogel P^2 column,using ethanol/water (2:8 v/v) as eluent.The yield of hexamer was 72.8% (Figure 3).

Preparation and purification of dodecanucleotide d(ACCACCACCACC) by phosphoramidite approach

5′-O-dimethoxytrityl-N^4-α-phenylcinnamoyl-2′-deoxycytidine−3′-O-succinate was linked to the support (LCAA/CPG) by usual procedure (24) . Loading was estimated to be 38 μmole/g. Functionalised dry resin (5 mg) was taken in a small cassette and after detritylation and usual wash cycle coupling was done with 5′-O-dimethoxytrityl-N^6-benzoyl 2′-deoxycytidine 3′-β-cyanoethyl-N,N-diisopropyl phosphoramidite (20 molar equiv), ^{1}H-tetrazole (25 molar equiv) in acetonitrile (10 min).It was then followed by washing with acetonitrile (2 min),oxidation with iodine in THF-2,6 lutidine-water (1 min), capping with acetic anhydride and 4-dimethylaminopyridine (0.5 min) and final washing with dichloromethane (2 min).The couplings and washings were repeated ten times with different nucleoside phosphoramidites to get the desired sequence.

The resin was treated with conc. ammonia at 50°C for 16 h to delink the dodecamer from support and simultaneously to remove the amino protecting groups.The partially deprotected dodecamer was purified first by passing it through C_{18} SEP-PAK cartridge and then after detritylation through Mono 'Q' anion exchange hplc column using 10mM NaOH and 10mM NaOH with 1 M NaCl as buffer A and buffer B respectively.Fraction containing dodecanucleotide was desalted by passing it through Biogel P^2 column using ethanol/water (2:8 v/v) as eluent.The yield of dodecamer was 76% (Figure 4).

Estimation of degree of depurination

Dilute solutions (15 μmole ml^{-1}) of N^6-α-PhCm-dA, N^6-bZ-dA,dA, N^2-α-PhCm-dG,N^2-ibu-dG and dG were prepared by dissolving 0.105 mmol of each in 7 ml solvent. Solvent used for N-protected nucleosides was dichloromethane and that for free nucleosides was water.The solutions were divided into seven equal parts.

Each fraction was treated with 80% acetic acid (2 ml) at r.t. Reactions were quenched by triethylamine-methanol (4:1 v/v) at varied time intervals.In case of dA and its derivatives

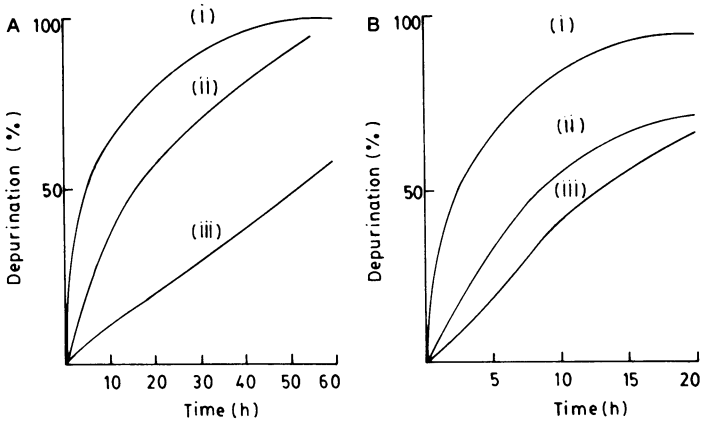

Fig.5 (A) Depurination of dA and its N-protected derivatives (i) N^6-bZ-dA,$t_{1/2}$ 4.5 h (ii) N^6-α-PhCmdA,$t_{1/2}$ 14.5 h (iii) dA,$t_{1/2}$ 50 h

Fig.5 (B) Depurination of dG and its N-protected derivatives (i) N^2-ibu-dG,$t_{1/2}$ 2.5 h (ii) N^2-α-PhCm-dG,$t_{1/2}$ 8.5 h (iii) dG,$t_{1/2}$ 13 h.

quenching time was 30 min, 1 h,2 h,4 h,6 h,20 h and 60 h and in the case of dG and its derivatives 15 min,30 min,1 h,2 h,4 h,10 h and 20 h.After quenching each fraction was evaporated to dryness and dissolved in minimum of solvent (dichloromethane for N-protected nucleosides and water for free nucleosides).These solutions were applied over preparative tlc.Solvent for tlc was dichloromethane/methanol (9.5:0.5v/v) for N-protected nucleosides and dichloromethane/methanol (8:2 v/v) for free nucleosides.Out of the two bands obtained,the upper band was depurinated part (free base) and lower was non-depurinated one. These bands were scratched and eluted with dichloromethane in case of N-protected derivatives and with water in case of free nucleosides. U.V. absorption was measured at wavelengths 288 nm for N^6-α-PhCmdA,280 for N^6-bZ-dA,260 for dA,284 for N^2-α-phCmdG, 260 for N^2-ibu-dG and 252 for dG.Total O.D. of each band was taken as its concentration and percentage of depurination was calculated (Figure 5).

RESULTS AND DISCUSSION

α-phenyl cinnamoyl group has been used for selective protection of exocyclic amino function of all the three deoxynucleosides i.e. dA,dG and dC (Figure 1A,B & C).

The very high selectivity observed during the protection of all the three deoxynucleosides is of utmost importance as it eliminates two step procedure of protection,whereby first a fully protected derivative is formed which on selective hydrolysis gives the desired product (Khorana's procedure) and eliminates the need of prior silylation (Jones procedure).Removal conditions of α-phenyl cinnamoyl group are comparatively milder than all other conventional groups used so far.

α-phenyl cinnamoyl derivatives, being hydrophobic in nature are highly soluble in organic solvents and are therefore well suited for solvent extraction,adsorption chromatography and for purification of oligomers by reversed phase hplc (25).

In order to adjudge the applicability of the group in oligonucleotide sysnthesis by solid phase method,a hexanucleotide d(AAGCTT) and a dodecanucleotide

d(ACCACCACCACC) were synthesized by phosphotriester and phosphoramidite approaches respectively. Good yields of oligomers obtained in each case, showing thereby more than 95% coupling yield declare the suitability of the group in solid phase synthesis.The unsaturated groups have been reported earlier (7) to be well suited for oligonucleotide synthesis.

The most exciting part of the study was the extrastability of α-phenylcinnamoyl derivatives,under acidic conditions, thereby minimising depurination.The N-α-PhCm derivatives of 2′-dA (t1/2, 14.5 h) and 2′-dG (t1/2,8.5 h) have been found to be much more stable than conventional N- protected nucleosides (t1/2,4.5 h & 2.5 h) respectively (Figure 5A & 5B).This undoubtedly explains the comparatively high yields of the desired oligomers obtained.

Acylation of amino function in purine nucleosides facilitates the protonation at N^7 (26) which makes glycosyl bond more prone to hydrolysis under acidic conditions (27−28).Since purine nucleosides exist in syn and antiform,the latter being more probable,the introduction of bulky group on amino function inhibits rotation of N-glycosyl linkage and places N^7 in the groove of 5′-OH and heterocyclic base.This is the reason behind using bulky groups for depurination resistivity (29).

Attempts have been made from time to time to introduce depurination resistant amino protecting groups but success obtained was mere compromising.An alternate procedure to use N^1-oxidised derivative was also adopted to check depurination in case of 2′-deoxyadenosine (30) but analogous procedure cannot be applied in the case of 2′-deoxyguanosine.We have found that α-phenylcinnamoyl group renders better depurination resistivity to the intermediates (half life N^6-α-PhCmdA, 14.5 h; N^2-α-PhCm-dG 8.5 h).The possible reasons of excellent depurination resistant nature of α-phenylcinnamoyl derivatives of 2′-dA and 2′-dG are as follows,(i) In case of α-PhCmdA, β-hydrogen atom of the α-phenyl cinnamoyl group by virtue of its weakly acidic nature may form a weak intramolecular hydrogen bond (C-H---N^7) with N^7 and thereby reduce the basicity of N^7 nitrogen atom making it less prone to the attack of proton during treatment with protonated acids. Whereas in case ofα-PhCmdG,the β-hydrogen atom of the α-phenyl cinnamoyl group may form a weaker hydrogen bond with N^1 Nitrogen (C-H---N^1) in the unusual tautomeric form of guanine,which may predominate for reasons of more stability acquired due to H-bonding and therefore may contribute to depurination resistivity by reducing the basicity of N^1 Nitrogen which is a potential protonation site in amino protected-2′-deoxyguanosine derivative. (ii) The bulky nature of the group prevents free rotation of the glycosyl bond and (iii) The stereospecific structure of α-phenyl cinnamoyl group having both benzene rings in cis-position (as confirmed by decarboxylation of α-phenylcinnamic acid by quinoline , copper chromium oxide yielding cis-stilbene and also by I.R. spectra of N-protected derivatives $IR_{(KBr)}$ 710 to 660 cm^{-1}) makes it comparatively more hydrophobic and restricts completely the approach of H_3O^+ at N^7 and N^1 nitrogen atoms.

Thus it can be stated that α-phenylcinnamoyl group by virtue of its high selectivity in case of all the three deoxynucleosides, comparatively high depurination resistant nature, milder deprotection conditions and high lipophilic nature stands as a promising protecting group in oligonucleotide synthesis.Moreover,the high selectivity of the group for amino function can be well exploited for protection of the corresponding monomers in ribo-series,where the problem is more acute because of an additional step involving selective protection of 2′-hydroxl group.The studies in this direction are in progress.

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